Form PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **702-002201**

U.S APPLICATION NO. (If known, see 37 CFR 1 5): 09/720933

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATES CLAIMED
PCT/NL99/00417	**02.07.99 (July 2, 1999)**	**02.07.98 (July 2, 1998)**

TITLE OF INVENTION **BONE CEMENT WITH ANTIMICROBIAL PEPTIDES**

APPLICANT(S) FOR DO/EO/US **Elisabeth H. BURGER, Arie VAN NIEUW AMERONGEN, Paulus I. J. M. WUISMAN**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S C 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U S C. 371

3. ☒ This express request to begin national examination procedures (35 U.S C 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date

5. ☒ A copy of the International Application as filed (35 U S C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S C 371(c)(2))

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U S.C 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
   b. ☐ have been transmitted by the International Bureau
   c. ☐ have not been made, however, the time limit for making such amendments has NOT expired
   d. ☒ have not been made and will not be made

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))

9. ☐ An oath or declaration of the inventor(s) (35 U S C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U S C 371(c)(5))

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1 98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3.31 is included

13. ☒ A FIRST preliminary amendment
    ☐ A SECOND or SUBSEQUENT preliminary amendment

14. ☐ A substitute specification

15. ☐ A change of power of attorney and/or address letter

16. ☒ Other items or information:
    a. WO 00/01427-Front Page with Abstract, specification and claims (15 pp.)
    b. Search Report (4 pp)
    c. International Preliminary Examination Report (4 pp)

526 Rec'd PCT/PTO 02 JAN 2001

U S APPLICATION NO (If known, see 37 CFR 1.5) 09/720933	INTERNATIONAL APPLICATION NO PCT/NL99/00417	ATTORNEY'S DOCKET NUMBER 702-002201

17 ☒ The following fees are submitted.

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		CALCULATIONS PTO USE ONLY
Search Report has been prepared by the EPO or JPO	$860.00	
International preliminary examination fee paid to USPTO (37 CFR 1 482)	$690.00	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$710.00	
Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO	$1000.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$100.00	

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	34 - 20	14	X **$18.00**	$ 252.00	
Independent claims	2 - 3 =	0	X **$80.00**	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 1242.00	
Reduction of 1/2 for filing by small entity, if applicable Small Entity Statement verified by Applicant(s) attorney.				$ 0.00	
SUBTOTAL =				$ 1242.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				$ 0.00	
TOTAL NATIONAL FEE =				$ 1242.00	
Fee for recording the enclosed assignment (37 CFR 1 21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3.31). **$40.00** per property +				$ 0.00	
TOTAL FEES ENCLOSED =				$ 1242.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$ 1242.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ____ in the amount of $ _____ to cover the above fees
A duplicate copy of this sheet is enclosed

c. ☒ The Assistant Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0650. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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PATENT APPLICATION/PCT
Attorney Docket No. 702-002201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	
Elisabeth H. BURGER, **Arie VAN NIEUW AMERONGEN,** **Paulus I. J. M. WUISMAN**	: : :	**BONE CEMENT WITH ANTIMICROBIAL PEPTIDES**
International Application No. PCT/NL99/00417	:	
International Filing Date 02 July 1999	:	
Priority Dates Claimed 02 July 1998	:	
Serial No. Not Yet Assigned	:	
Filed Concurrently Herewith	:	

Pittsburgh, Pennsylvania
January 2, 2001

PRELIMINARY AMENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Prior to initial examination, please amend the above-identified patent application as follows:

Page 1, after the title, insert the following headings:

--BACKGROUND OF THE INVENTION

1. Field of the Invention--.

Page 1, after line 5, insert the following heading:

--2. Description of the Prior Art--.

Page 1, after line 23, insert the following heading:

--SUMMARY OF THE INVENTION--.

Page 3, line 12, before "positions" insert --odd-numbered--.

Page 3, line 14, before "positions" insert --even-numbered--.

Page 4, after line 8, insert the following heading:

--DESCRIPTION OF THE PREFERRED EMBODIMENTS--.

Page 7, line 31, delete "en" and substitute therefor --and--.

IN THE CLAIMS:

Please cancel claims 1-34 and rewrite them as new claims 35-68 as follows:

--35. Bone material for the prevention and treatment of osteomyelitis, which material is provided with antimicrobial peptides (AMPs) consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain is positively charged amino acids and the majority of the amino acids of the other half of the domain is uncharged amino acids, which AMPs can be released to the surrounding area for a period of time and wherein the bone material forms bone cement after curing and the AMPs are distributed homogeneously in the cured bone cement.

36. The bone material as claimed in claim 35, wherein the domain forms an α-helix and at least at a majority of the positions 1, 2, 5, 6, 9, and positions 12, 13, 16, 19, 20, 23 and 24 if present contains a positively charged amino acid, at position 8 a positive or an uncharged amino acid and at least at a majority of the positions 3, 4, 7, 10, and positions 11, 14, 15, 17, 18, 21, 22, 25 if present contains an uncharged amino acid.

37. The bone material as claimed in claim 36, wherein the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

38. The bone material as claimed in claim 36, wherein the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

39. The bone material as claimed in claim 36, wherein the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

40. The bone material as claimed in claim 36, wherein the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

41. The bone material as claimed in claim 36, wherein the domain makes up the entire peptide.

42. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

KRLFKELKFSLRKY (peptide 3).

43. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

KRLFKELLFSLRKY (peptide 4).

44. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

KRLFKELKKSLRKY (peptide 5).

45. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

KRLFKELLKSLRKY (peptide 6).

46. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

OOLFOELOOSLOOY (peptide 7).

47. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

OOLFOELLOSLOOY (peptide 8).

48. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

KRLFKKLKFSLRKY (peptide 9).

49. The bone material as claimed in claim 36, wherein the domain has the following amino acid sequence:

KRLFKKLLFSLRKY (peptide 10).

50. The bone material as claimed in claim 35, wherein the domain forms an α-helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 a positively charged amino acid.

51. The bone material as claimed in claim 35, wherein the domain forms an α-helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains a positively charged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 an uncharged amino acid.

52. The bone material as claimed in claim 58, wherein the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

53. The bone material as claimed in claim 50, wherein the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

54. The bone material as claimed in claim 58, wherein the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

55. The bone material as claimed in claim 50, wherein the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

56. The bone material as claimed in claim 50, wherein the domain makes up the entire peptide.

57. The bone material as claimed in claim 50, wherein the domain has the following amino acid sequence:

LLLFLLKKRKKRKY (peptide 11).

58. The bone material as claimed in claim 35, wherein the domain forms a so-called β-strand and contains a positively charged amino acid on at least a majority of the odd-numbered positions 1, 3, 5, 7, 9, and positions 11, 13, 15, 17, 19, 21, 23 and 25 if present and an uncharged amino acid on at least a majority of the even-numbered positions 2, 4, 6, 8, 10, and positions 12, 14, 16, 18, 20, 22, 24 if present.

59. The bone material as claimed in claim 58, wherein the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

60. The bone material as claimed in claim 58, wherein the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

61. The bone material as claimed in claim 58, wherein the majority of the positively charged amino acids is the total number of odd-numbered positions minus 1.

62. The bone material as claimed in claim 58, wherein the majority of the uncharged amino acids is the total number of even-numbered positions minus 1.

63. The bone material as claimed in claim 24, wherein the domain makes up the entire peptide.

64. The bone material as claimed in claim 35, wherein the N-terminus is amidated.

65. The bone material as claimed in claim 35, wherein the C-terminal carboxylic acid group is replaced by an amide, ester, ketone, aldehyde or alcohol group.

66. The method of manufacturing bone material as claimed in claim 35, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement.

67. The method as claimed in claim 66, wherein the AMPs are dissolved in liquid medium, preferably water, and mixed with the bone material after curing thereof.

68. The method as claimed in claim 66, wherein the cured bone cement is formed to a granulate.--

IN THE ABSTRACT:

After the claims, please insert a page containing the Abstract Of The Disclosure, which is attached hereto as a separately typed page.

REMARKS

The specification has been amended by this Preliminary Amendment to place the application in conformance with standard United States patent practice.

Original claims 2-34 have been canceled by this Preliminary Amendment and rewritten as new claims 35-68 in order to conform the claims to standard United States patent practice.

A further Amendment will be submitted in due course to address the amino acid sequence listings.

An Abstract of the Disclosure has been added as a separately typed page to be inserted after the claims.

Entry of this Preliminary Amendment and examination and allowance of claims 35-68 are respectfully requested.

Respectfully submitted,

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BONE CEMENT WITH ANTIMICROBIAL PEPTIDES

The invention relates to the use of antimicrobial peptides (AMP) in calcium phosphate bone cement and forms a system which provides for slow release of the AMP for prevention and treatment of infections of the bone (osteomyelitis) and the surrounding soft tissues.

Preventing infections of the soft tissues and the bone after operations remains a cause for concern in orthopaedic and trauma surgery. Infection of bone tissues (osteomyelitis) and/or the surrounding soft tissue is very difficult to cure and this is a reason why stringent prevention is required. At this moment granules of polymethyl methacrylate (PMMA-granules) are used for this purpose. When they are placed in the surgical wound they function as a slow release system for obtaining high local concentrations of antibiotics, while the systemic concentrations remain low. Such granules are however non-re-absorbable and an additional operation is therefore necessary. The intensive use of antibiotics in human and veterinary medicine has further resulted in large scale resistance of bacteria and fungi to antibiotics such as gentamicin. New therapies for prevention and treatment of for instance osteomyelitis are therefore urgently required.

The present invention provides for this purpose a new system for the prevention and treatment of osteo-myelitis, which makes use of a re-absorbable calcium phosphate cement carrier and a new class of antibiotic agents, the so-called antimicrobial peptides (AMPs).

The AMPs used in the invention are peptides consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively

charged amino acids and the majority of the other half of the domain are uncharged amino acids.

The structure of these peptides has a number of variations. Firstly, the domain can form an α-helix, of which at least a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains a positively charged amino acid, position 8 is a positive or an uncharged amino acid and at least a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains an uncharged amino acid. These peptides have a lateral amphipathicity, i.e. a maximum hydrophobic moment at 100°. Stated simply, these peptides are hydrophobic on the left side and hydrophilic on the right side or vice versa. These peptides are referred to herein as "type I".

The domain can further form an α-helix, of which at least a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains an uncharged amino acid, position 8 is a positive or an uncharged amino acid and at least a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains a positively charged amino acid. These peptides have a lateral amphipathicity, i.e. a maximum hydrophobic moment at 100°. Stated simply, these peptides are hydrophobic on the right side and hydrophilic on the left side or vice versa. These peptides are designated "type II" herein and are in principle mirror-symmetrical to type I peptides.

In addition, the domain can form an α-helix, wherein at least a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and a positively charged amino acid is found at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25. These peptides have a longitudinal amphipathicity, i.e. a minimum hydrophobic moment at 100°. These peptides are hydrophobic on their "top" and hydrophilic on their "bottom". Such peptides are designated "type III".

Conversely, the domain can form an α-helix, wherein at least a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains a positively charged amino acid and an uncharged amino acid is found at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25. These peptides likewise have a longitudinal amphipathicity and therefore a minimum hydrophobic moment at 100°. These peptides are hydrophobic on their "bottom" and hydrophilic on their "top". Such peptides are designated "type IV".

Finally, the domain can form a so-called ß-strand and contain a positively charged amino acid on at least a majority of the positions 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 and 25) and an uncharged amino acid on at least a majority of the positions 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24). Such a ß-strand is laterally amphipathic and has a maximum hydrophobic moment at 180°. The ß-strand structure is flatter than the α-helix and, stated simply, is hydrophobic on the left and hydrophilic on the right or vice versa. These are "type V" peptides.

The positively charged amino acids are preferably chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H), while the uncharged amino acids are preferably chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W). Amino acids on the border between hydrophilic and hydrophobic can be chosen from both groups or from the remaining amino acids.

Hardly any difference in activity can in principle be detected when one of the positive amino acids and/or one of the uncharged amino acids is replaced by a random amino acid. The majority of the positively charged amino acids is therefore preferably the total number of

positively charged amino acids minus 1 and the majority of the uncharged amino acids is preferably the total number of uncharged amino acids minus 1.

The domain can be a part of a larger peptide but can itself also make up the entire peptide. When the domain forms part of a larger peptide, the C-terminal and/or N-terminal amino acids which are then additionally present can be random amino acids.

The following peptides of the type I are particularly recommended:

KRLFKELKFSLRKY (peptide 3)
KRLFKELLFSLRKY (peptide 4)
KRLFKELKKSLRKY (peptide 5)
KRLFKELLKSLRKY (peptide 6
OOLFOELOOSLOOY (peptide 7)
OOLFOELLOSLOOY (peptide 8)
KRLFKKLKFSLRKY (peptide 9)
KRLFKKLLFSLRKY (peptide 10)

A preferred peptide of the type III has the following amino acid sequence:

LLLFLLKKRKKRKY (peptide 11)

The peptides according to the invention can also contain further modifications. These modifications are for instance an N-terminal amide ring, for instance with acetic acid anhydride, or an alternative cleavage of the synthesis resin by which the C-terminus is modified. For this latter a replacement of the C-terminal carboxylic acid group by an amide, ester, ketone, aldehyde or alcohol group can be envisaged. Peptides with such a modification are for instance:

KRLFKELKFSLRKY-amide (peptide 12)
KRLFKELLFSLRKY-amide (peptide 13)

In addition to single peptides, oligomers can also be made. These are preferably linear oligomers of the peptides according to the invention. The coupling can be head-to-head and tail-to-tail as well as head-to-tail,

either by direct synthesis or by post-synthetic enzymatic coupling. For a trans-membrane pore formation a minimum peptide length is required. Oligomers of the peptides according to the invention are double length and thereby better able in principle to span the whole phospholipid double layer of the bacterial cell membrane at one time. The activity of the peptide could hereby improve even further. In addition, extension of the peptides provides stabilisation of the helix conformation. A spacer must usually be inserted. In direct synthesis of head-to-tail coupled oligomers a spacer can be inserted to size by the use of a chain of unnatural amino acids of the correct length, for instance ß-alanine, γ-amino butyric acid, ε-amino caproic acid, etc. Heterodifunctional coupling reagents, such as are commercially available for coupling peptide antigens to carrier proteins (for instance 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), m-maleimidobenzoyl)-N- hydroxysuccinimide ester (MBS), N-succinimidyl 3-[pyridyldithio]propionate (SPDD) etc.) are used to make linear oligomers with an inserted spacer. For head-to-head and tail-to-tail couplings can be used trivalent amino acids such as asparagine acid (D), glutamine acid (E), ornithine (O), lysine (K), serine (S), cysteine. Such oligomers are for instance:

KRKFHEKHHSHRGYC-CYGRHSHHKEHFKRK (peptide 14)

YGRHSHHKEHFKRKC-CKRKFHEKHHSHRGY (peptide 15)

$^{\alpha}N,^{\varepsilon}N$-(KRKFHEKHHSHRGY)$_2$K-amide (peptide 16)

$^{\alpha}N,^{\varepsilon}N$-(KRLFKELKFSLRKY)$_2$K-amide (peptide 17)

$^{\alpha}N,^{\varepsilon}N$-(KRLFKKLKFSLRKY)$_2$K-amide (peptide 18)

Peptides 14 and 15 are obtained by synthesis of peptide 2 with an additional C-terminal respectively N-terminal cysteine, whereafter the oligomer is obtained by air oxidation. Peptides 16, 17 and 18 are obtained by making use of the Multiple Antigenic Peptide (MAP) strategy, wherein a lysine having on both the α- and on the ε-amino group an Fmoc protection was used as first amino acid on

the synthesis resin, whereby two identical amino acid chains (peptides 2, 3 and 9) were synthesized simultaneously on one lysine molecule.

The peptides described herein have no or hardly any haemolytic activity in physiological buffers such as PBS (phosphate-buffered saline solution). A low activity against erythrocytes of human origin is an indication of low toxicity. This selectivity is essential for the use of these peptides as antibiotics.

The peptides have a wide spectrum of antibacterial and antifungal activity, even against methycillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa (which is particularly dangerous in the case of osteomyelitis) and amphotericin-B-resistant Candida albicans.

The invention further makes use of bone material which after curing forms bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement. It is biocompatible, re-absorbable and inert, and forms at body temperature. The final cement moreover has sufficient strength and stiffness to serve as bone replacement.

It has been found according to the invention that the inclusion of the AMPs in the cement does not affect the mechanical properties thereof.

In order to include the AMPs in the cement, they are dissolved in a liquid medium, preferably water, and mixed with the bone material before or after curing thereof.

A blood protein-containing solution, in particular albumin, is preferably used to hold the AMPs in solution, in order to ensure a homogeneous distribution of the AMPs in the final cured bone cement.

In a preferred embodiment bone material contains calcium phosphate. With a view to the biocompatibility this is particularly a mixture of dicalcium phosphate,

tricalcium phosphate, tetracalcium phosphate and/or hydroxyl-apatite.

The invention further relates to a method of manufacturing a bone material according to the invention, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement. As stated, the AMPs are dissolved in a liquid medium, preferably water, and mixed with the bone material before or after curing thereof. The AMPs are preferably mixed with the bone material after curing. A longer release period is thus provided in which the AMPs can be released to the surrounding area after arranging of the bone material. The starting point here in each case is that the AMPs are always active only where this is necessary.

The invention also relates to a device for administering bone material provided with AMPs according to the invention, wherein provision is made for at least two compartments for separately containing the bone material and AMPs, a mixing chamber for mixing the bone material and the AMPs and a spray nozzle for spraying the mixture out of the mixing chamber.

The invention will be further elucidated with reference to a discussion of a number of tests in accordance with preferred variants of the invention, wherein the procedures for manufacturing the present bone material with added AMPs will be discussed.

1. A sterile cement powder consists of a mixture of alpha-tricalcium phosphate, tetracalcium phosphate-monoxide en dicalcium phosphate dibasic in a ratio of 75:20:5, or otherwise if desired.

2. A sterile AMP solution (solution (A)) consists of 4 mM HCl in water having dissolved therein 0.1% beef

or human serum albumin and AMPs in a concentration as required varying from $2x10^{-5}$% to 2%.

3. A second sterile solution (solution (B)) consists of water having dissolved therein 12% sodium succinate and 5% chondroitin succinate.

4. Solution (A) is mixed 1 to 1 with solution (B) under sterile conditions.

5. One volume part solution (A+B) is mixed with two volume parts cement powder under sterile conditions. This can take place:

   a. in a dish and mixed with a spatula, whereafter the cement paste is arranged immediately in-situ in the body of the patient and there cures;

   b. via a spray with two chambers, one of which contains the cement powder and the other solution (A+B); using the spray, powder and liquid are brought together in-situ in the body, whereafter the mixture cures at this location.

   c. in a dish, mould or container, whereafter the mixture cures outside the body and is optionally ground to a powder of the desired granule size, whereafter it is arranged in the body of the patient.

6. One volume part solution B is mixed with two volume parts cement powder under sterile conditions in a dish, mould or container, whereafter the mixture cures and is ground to a

powder of the desired granule size. The cured cement is then incubated for 1 or more hours in solution A, whereafter the cement with absorbed AMPs is dried and stored in dry form until it is arranged in the body of the patient.

CLAIMS

1. Bone material for the prevention and treatment of osteomyelitis, which material is provided with antimicrobial peptides (AMPs) consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged amino acids and the majority of the amino acids of the other half of the domain are uncharged amino acids, which AMPs can be released to the surrounding area for a period of time and wherein the bone material forms bone cement after curing and the AMPs are distributed homogeneously in the cured bone cement.

2. Bone material as claimed in claim 1, **characterized in that** the domain forms an α-helix and at least at a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains a positively charged amino acid, at position 8 a positive or an uncharged amino acid and at least at a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains an uncharged amino acid.

3. Bone material as claimed in claim 2, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

4. Bone material as claimed in claim 2 or 3, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

5. Bone material as claimed in claims 2-4, **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

6. Bone material as claimed in claims 2-5, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

7. Bone material as claimed in claims 2-6, **characterized in that** the domain makes up the entire peptide.

8. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELKFSLRKY (peptide 3).

9. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELLFSLRKY (peptide 4).

10. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELKKSLRKY (peptide 5).

11. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELLKSLRKY (peptide 6).

12. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

OOLFOELOOSLOOY peptide 7).

13. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

OOLFOELLOSLOOY (peptide 8).

14. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKKLKFSLRKY (peptide 9).

15. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKKLLFSLRKY (peptide 10).

16. Bone material as claimed in claim 1, **characterized in that** the domain forms an α-helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 a positively charged amino acid.

17. Bone material as claimed in claim 1, **characterized in that** the domain forms an α-helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains a positively charged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 an uncharged amino acid.

18. Bone material as claimed in claim 16 or 17, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

19. Bone material as claimed in claim 16, 17 or 18, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

20. Bone material as claimed in claims 16-19, **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

21. Bone material as claimed in claims 16-20, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

22. Bone material as claimed in claims 16-21, **characterized in that** the domain makes up the entire peptide.

23. Bone material as claimed in claims 16 and 18-22, of which the domain has the following amino acid sequence:

LLLFLLKKRKKRKY (peptide 11).

24. Bone material as claimed in claim 1, **characterized in that** the domain forms a so-called ß-strand and contains a positively charged amino acid on at least a majority of the positions 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 and 25) and an uncharged amino acid on at least a majority of the positions 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24).

25. Bone material as claimed in claim 24, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

26. Bone material as claimed in claim 24, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

27. Bone material as claimed in claims 24-26, **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

28. Bone material as claimed in claims 24-27, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

29. Bone material as claimed in claims 24-28, **characterized in that** the domain makes up the entire peptide.

30. Bone material as claimed in claims 1-29, wherein the N-terminus is amidated.

31. Bone material as claimed in claims 1-30, wherein the C-terminal carboxylic acid group is replaced by an amide, ester, ketone, aldehyde or alcohol group.

32. Method of manufacturing bone material as claimed in any of the claims 1-31, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement.

33. Method as claimed in claim 32, wherein the AMPs are dissolved in liquid medium, preferably water, and mixed with the bone material after curing thereof.

34. Method as claimed in claim 32 or 33, wherein the cured bone cement is formed to a granulate.

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BONE CEMENT WITH ANTIMICROBIAL PEPTIDES

ABSTRACT OF THE DISCLOSURE

The invention relates to bone material for the prevention and treatment of osteomyelitis, which material is provided with antimicrobial peptides (AMPs) consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain is positively charged amino acids and the majority of the amino acids of the other half of the domain is uncharged amino acids, which AMPs can be released to the surrounding area for a period of time and wherein the bone material forms bone cement after curing and the AMPs are distributed homogeneously in the cured bone cement. The invention further relates to a method of manufacturing the bone material, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement.

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Bone cement with antimicrobial peptides

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 2 July 1999 as PCT/NL99/00417 as

Application Serial No. 09/720,933 received on January 2, 2001

and was amended on January 2, 2001 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
98202233.7 (Number)	Europe (Country)	2 July 1998 (Day/Month Year Filed)	☒ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*list name and registration number*)

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